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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

Hepatocyte growth factor (HGF) binding to its transmembrane receptor, Met, results in an increase in breast epithelial cell motility, morphogenesis and metastasis. HGF-like forms have been detected as secretion products of the human breast epithelial carcinoma cell line, MCF10A1T3B. The two major products are ~56 and ~32 kDa. Recombinant HGF treatment with elastase produced the ~56 kDa HGF antagonistic product, an NK4-like form. Anion exchange chromatography of conditioned medium indicated that MCF10A1T3B secretion products have decreased affinity compared to endogenous full-length HGF. The secretion products bind to Met, as detected by ELISA, but are unable to induce cell scatter, as detected by MDCK scatter assay. ELISA detection of HGF-Met binding was also used to screen other potential modulators of this interaction. HGF-Met binding occurred within the pH 6-7 range. The lysine analogue, ϵ-ACA, at a concentration as high as 100 mM, had no effect on HGF-Met binding. This binding was also completely abrogated by concentrations of $ZnCl_2$ and $CuCl_2$ as low as 2.5 mM, while the same concentration of $MnCl_2$ slightly enhanced binding. A concentration of 50 μM $CuCl_2$ altered HGF mobility in a non-denaturing gel, while 1 mM $MnCl_2$ had no effect. $CuCl_2$, at 500 μM and 1 mM, inhibited HGF-induced cell scatter and Met phosphorylation, respectively.

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Introduction:

Breast cancer is one of the leading causes of death in women, with 1 in every 9 North American women developing breast cancer in their lifetime (1,2). Incidences of breast cancer are positively correlated with a number of factors, such as increased age, increased age of menopause, decreased age of menarche, smoking and obesity. Rates of detected breast cancer have been steadily increasing over the last five decades, while mortality rates have slightly declined. This is partially due to a better understanding of breast cancer etiology, earlier and better detection methods, and improved therapeutic approaches therefore, emphasizing the importance of continued research in helping to reduce breast cancer deaths.

As cancer research has progressed, a number of proteins have been identified as being contributors to this disease. Often these proteins are either up-regulated or aberrant and act in conjunction with one another to cause the debilitating effects. One such protein studied in our laboratory is hepatocyte growth factor (HGF) which exhibits its effects through interaction with its receptor Met. HGF is secreted by stromal cells in the breast as an immature pro-HGF, which can undergo processing by a number of factors such as urokinase-type plasminogen activator (uPA), tissue-type plasminogen activator (tPA), HGF-converting enzyme or thrombin (3,4,5,6,7). Mature HGF consists of a 69 kDa α-chain and a 34 kDa β-chain (8,9). The HGF α-chain is composed of an N-terminal hairpin loop (N), followed by four kringle domains (K1, K2, K3, K4), with each kringle domain containing three intra-molecular disulfide bonds. Following the four kringle domains and located at the C-terminus of the α-chain, is a disulfide bond linking the α– and β-chains of HGF. The HGF receptor, known as Met, is a transmembrane protein expressed on the surface of epithelial cells in the breast (10,11). Met also consists of two disulfide-linked chains, a 50 kDa extracellular α-chain and a 140 kDa transmembrane β-chain. The intracellular domain of Met contains phosphorylation sites which interact with downstream SH2-domain proteins, such as Grb2, Gab1, ERK2, and PI-3K (12,13,14). The activation of Met, by its extracellular interaction with HGF, causes an up-regulation of these downstream proteins, resulting in cellular changes such as increased motility, proliferation and morphogenesis (15, review). The extracellular Met α-chain interacts with full-length HGF, but the HGF isoforms, NK1 and NK2, are also able to bind to Met (16,17). These isoforms lack complete HGF activity, but yield insight into HGF-Met interaction domains and can serve as antagonists of full-length HGF. My project is to specifically investigate the interaction between HGF and Met and to screen potential inhibitors of this binding.

Two earlier observations obtained in our laboratory provide the basis for the current study. First, a human breast carcinoma cell line (MCF10A1T3B) over-expressing the *Ha-ras* oncogene has been shown to express aberrant HGF forms, compared to a non-malignant human breast epithelial control cell line (MCF10A1). MCF10A1T3B can be used to study the expression and function of aberrant HGF forms as possible mediators of HGF-Met binding. Identifying putative HGF fragments/domains involved in Met binding will be useful in the development of small peptide mimics. This information can be used to identify key amino acids or amino acid sequences of HGF binding epitopes for Met.

Second, recombinant HGF (rHGF) can be purified utilizing immobilized Cu(II)-affinity chromatography (18). Since the K1 domain of HGF contains two histidine residues in the putative Met binding region, we hypothesized that Cu(II) binding could potentially modulate HGF-Met binding and function. We therefore have studied the properties of Cu(II) binding to HGF as a possible lead to compounds that would alter HGF-Met interaction.

Body:

Original Hypothesis and Objectives:

Our original hypothesis was that HGF interaction with, and subsequent activation of Met, is a major step in transition of epithelial cells to a malignant phenotype. Hence, disruption of this interaction may play an important role in inhibiting this functions.

Two overall objectives were proposed:

1) **To identify, and assess the structural properties, of native HGF and putative HGF fragments secreted by breast carcinoma cell lines and tissues; then to further determine the effects of putative HGF fragments on Met activation and function in breast epithelial and carcinoma cells, and**

2) **To design high affinity peptides that either inhibit or promote the interaction between HGF and Met and to analyze these peptides in the modulation of HGF-Met function.**

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Objective 1:

A comparison of the morphology of MCF10A1 and MCF10A1T3B cell lines is shown (Figure 1). The MCF10A1T3B cell line shows increased cell scattering, filopodia and lamellipodia extensions, and increased cell growth rate, characteristic of a transformed phenotype (*Ha*-Ras transformed). Immunoassays were performed to identify secretion products of the two cell lines. Our lab has previously shown that full-length rHGF can be purified using Cu(II)-affinity chromatography and identified with subsequent western blotting and HGF immunoreactivity (18). This same technique was utilized in isolating putative HGF fragments (56 kDa and 32 kDa) from MCF10A1T3B conditioned media (CM) (Figure 2). Interestingly, these fragments have mobilities similar to that of HGF isoforms NK4 and NK2, respectively. We have subsequently attempted various methods in assaying the CM from the MCF10A1T3B cell line in order to characterize these fragments.

We have been able to detect the 56 kDa and 32 kDa fragments following concentration of the CM, without prior Cu(II)-affinity chromatography. A diethelaminoethylacetate (DEAE)-cellulose cation exchange column has been used to analyze binding and charge properties of the fragments. MCF10A1T3B CM was passed over the DEAE-cellulose column, with the putative HGF fragments eluting in low salt washes (0-0.3 M), while a band corresponding to the α-chain of HGF remained bound to the column at high salt concentrations (up to 1 M) (Figure 3). Using an enzyme-linked immunosorbant assay (ELISA), we were able to detect binding of a Met-IgG fusion protein (19) to these fragments, indicating that the purification step did not disrupt binding properties (data not shown).

Elastase has been implicated in post-translational modification of HGF to produce the NK4-like isoform (20). Therefore, we incubated rHGF in the presence of elastase, resulting in the appearance of a 56 kDa band (Figure 4). This finding suggests the 56 kDa band may be related to the NK4 isoform.

The 56 kDa and 32 kDa putative HGF fragments isolated from MCF10A1T3B CM were tested in a standard cell motility assay (21). The fragments did not induce cell scatter, indicating that these fragments lack complete HGF activity. The fragments will continue to be isolated and analyzed in competition assays with active rHGF.

Future analysis will examine potential inhibition, by these fragments, of HGF-induced cell scattering. Also, cells with HGF-inducible Met phosphorylation can be used to test the ability of the HGF fragments to induce or inhibit Met phosphorylation. Further binding studies will also be performed to assess affinity coefficients.

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Objective 2:

To begin screening inhibitor candidates of HGF-Met binding, we developed an ELISA system that efficiently measured binding of HGF to immobilized Met, with immobilized human IgG as a control. Various conditions and candidate compounds have been screened for induction or inhibition of HGF-Met binding. We have shown that HGF binding to Met occurs in the pH 6-7 range, but is completely abrogated at higher or lower pH conditions (Figure 5), suggesting that HGF-Met interaction is impaired under pH conditions varying from the human physiological range. Also, 0.1 M $CuCl_2$, the equivalent concentration used in HGF purification, inhibited HGF-Met binding (Figure 6). An equal concentration, 0.1 M, of the lysine analogue ε-ACA showed no inhibition of binding (Figure 6). We also found that as low as 1 mM $CuCl_2$ partially impeded binding, while 2.5 mM $CuCl_2$ totally abolished binding (Figure 7). HGF-induced cell scatter was inhibited by $CuCl_2$ (data not shown), as was HGF-induced tyrosine phosphorylation of Met (Figure 8). HGF mobility, but not Met-IgG mobility, in a non-denaturing agarose gel was shown to shift upward upon addition of $CuCl_2$, indicating a change in structure or charge due to Cu(II) binding (Figure 9).

Since a recent study has shown that HGF can be purified using polyvinyldiene fluoride-based immobilized metal affinity membranes (22), we screened other divalent cations for potential modulation of HGF-Met interaction. At a concentration each of 2.5 mM, $ZnCl_2$ had an effect similar to $CuCl_2$, while $MnCl_2$ enhanced HGF-Met binding (Figure 10).

We will further characterize and assess the nature of Cu(II) binding to HGF, as well as other potential metal ions exhibiting HGF or Met binding affinities. We will use NK1 and NK2 isoforms in future binding experiments, to localize Cu(II) binding domains. Also, the development of an efficient assay for HGF-Met binding, will allow the screening of short "phage display" peptides that bind to Met or HGF, according to the **original Objective 2.** Once these peptides are isolated, we can utilize competitive binding, scatter, tyrosine phosphorylation of Met, gel motility, as well as other assays to identify their function in HGF-Met interaction.

Key Research Accomplishments:

- Isolation and partial characterization of aberrant HGF secretion products from the MCF10A1T3B breast carcinoma cell line. The putative HGF fragments bind to Met, but no activity has been identified.

- Development of an efficient ELISA system to identify inhibitors of HGF binding to Met. This assay will also be used to identify HGF-Met antagonistic peptides.

- Demonstration that Cu(II) inhibits HGF binding to Met, HGF-induced scatter, HGF-induced tyrosine phosphorylation of Met and affects HGF conformation. Cu(II) may have a potential therapeutic role in the down-regulation of HGF activity and subsequent cellular processes, specifically in carcinomas.

- Identification of another divalent cation, Zn(II), which inhibits HGF-Met binding. This phenomenon requires further characterization.

Reportable Outcomes:

- Supervised two undergraduate students on this project:
  4th year thesis project (2001-2002): "Characterization of an Aberrant Form of Hepatocyte Growth Factor and its Role in Invasive Human Breast Carcinoma", Robert Herrington
  -summer student position (2002), Julie Tsai

- Abstract and poster presentation: "Hepatocyte Growth Factor Interaction with its Receptor, Met" (see appendix) accepted for the Era of Hope Meeting, Orlando, Florida, USA

- Manuscript in preparation: "Effect of Cu(II) on Hepatocyte Growth Factor (HGF) binding to its Receptor, Met: Potential Therapeutic Role in Breast Cancer", T.G. Wright, J. Tsai, Z.Jia, and B.E. Elliott

Conclusions:

Putative HGF fragments secreted by human breast carcinoma cells can be partially purified and assayed for their role in this malignancy. They have been shown to bind Met, but do not possess cell scattering activity. The Met binding ability of the secretion products, while lacking complete HGF activity, could indicate a modulating role of the secretion products in the mechanism of malignancy. Further functional assays, such as cell scattering, tyrosine phosphorylation of Met and cell invasion, in the absence or presence of rHGF, will be performed. Binding of HGF and Met can be measured and potential modulating conditions assessed.

HGF and Cu(II) exhibit binding affinity, as previously indicated by Cu(II)-sepharose affinity purification of HGF, and Cu(II) is able to inhibit HGF-Met binding and Met stimulation. Within a physiological concentration range, $CuCl_2$ has been shown to bind to HGF. Therefore, Cu(II) may serve as a potential therapeutic compound in regulating HGF stimulation of Met and subsequent downstream activities. Peptide mimics, alone or in conjunction with Cu(II), may also serve as modulators of HGF-Met binding.

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Figure 1. Transforming effect of *Ha-Ras* on morphology of human breast epithelial cells. A) A normal human breast epithelial cell line (designated MCF10A1) showed cell spreading with cell-cell contacts. **B)** *Ha-Ras*-transfected MCF10A1 cells (designated MCF10A1T3B) showed increased filopodia and lamellipodia formation and cell scattering. (200x magnification)
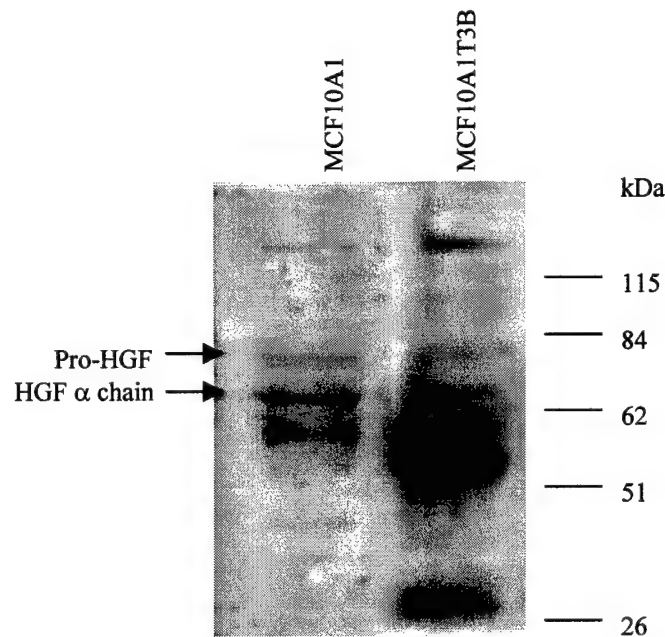

Figure 2. HGF expression status of MCF10A1 breast epithelial cells. CM from MCF10A1 and MCF10A1T3B cell lines were purified using Cu (II) affinity chromatography. The eluted fractions were then assessed for HGF expression by subjection to reduced 9% SDS-PAGE and blotting with rabbit anti-human HGF IgG. MCF10A1T3B CM showed increased presence of HGF (α chain), as well as two lower Mr HGF immunoreactive bands (56 kDa and 32 kda).
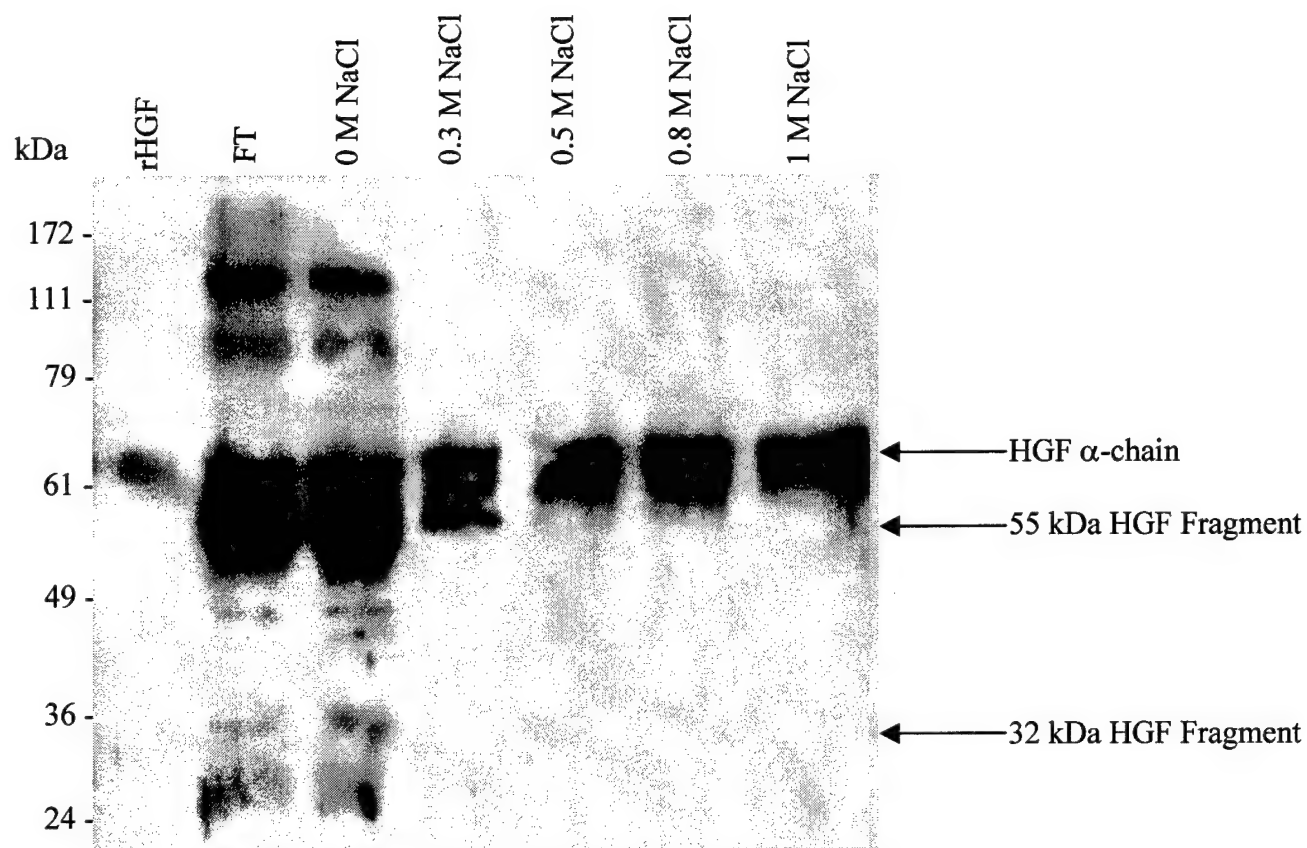
Figure 3. Western Blot Analysis of the Eluted Fractions of MCF10A1T3B Conditioned Media Through a DEAE-Cellulose Anion Exchange Column. The HGF α-chain was detected at 65 kDa in each elution fraction, indicating the HGF is immobilized in the column matrix. The elution of the 56 kDa HGF fragment by the 0.3 M NaCl elution fraction and the 32 kDa HGF fragment elution in the flowthrough (FT), indicates that these fragments are unable to bind tightly to the column. Increasing concentrations of NaCl were used as elution buffer.
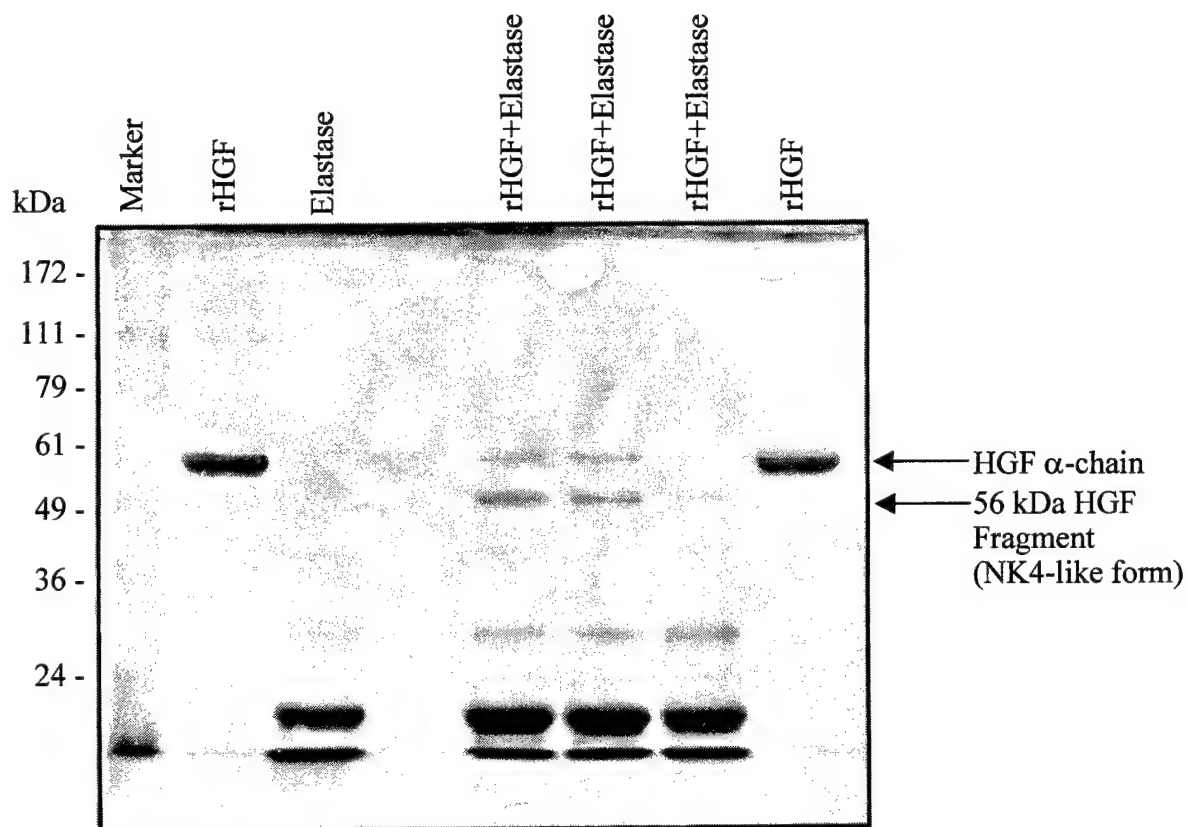
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Figure 4. Effect of Elastase on rHGF. Elastase (0.12 g) and rHGF (0.1 μg) were incubated in HBS buffer for 16 hrs at 25°C, then subjected to a 10% SDS-PAGE and Coomassie blue staining. The HGF α-chain was detected at 65 kDa in the rHGF control lanes. The lanes containing rHGF and elastase contained a reduced amount, compared to control lanes, of the 65 kDa HGF α-chain, as well as the presence of a 56 kDa HGF product. This 56 kDa fragment was not detected in the rHGF, or elastase controls.
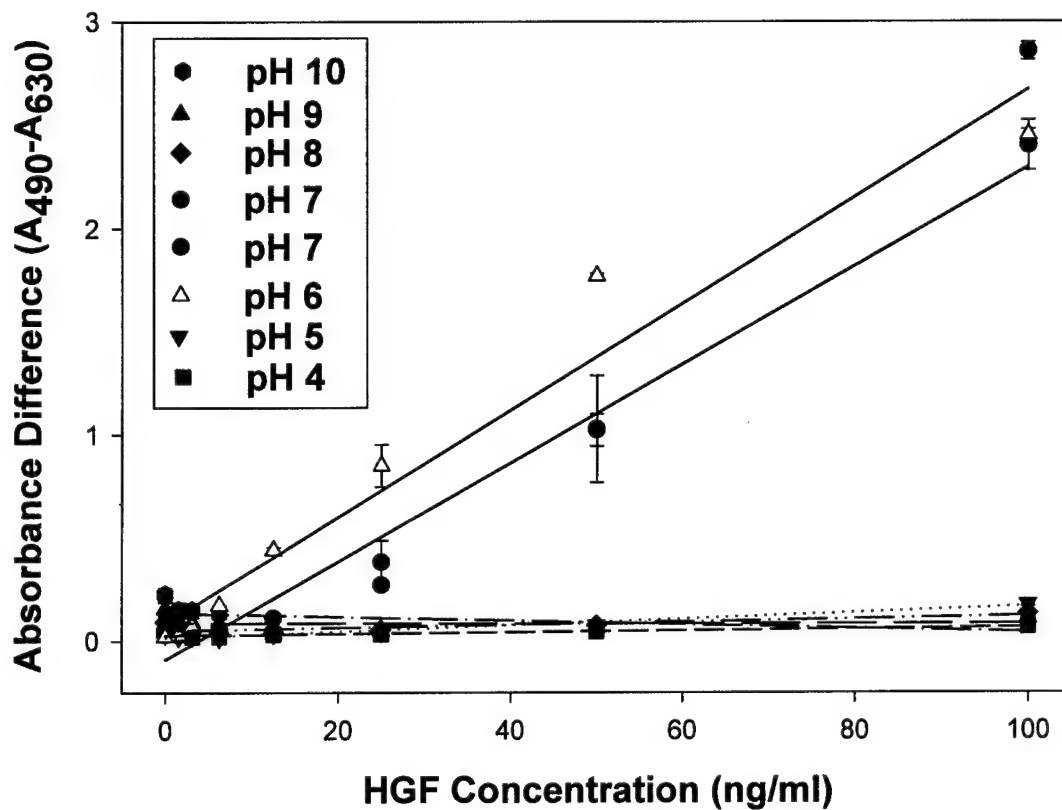
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Figure 5. Effect of pH on HGF binding to immobilized Met-IgG. Met-IgG was immobilized on an ELISA plate, followed by incubation with HGF at varying pH levels. HGF binding was measured by detecting HGF with a monoclonal anti-HGF IgG, followed by anti-murine HRP IgG and colour detection. The pH levels in which binding occurred were pH 7 (both closed circles) and pH 6 (open triangle), while the lower levels pH 5 (solid upside-down triangle) and pH 4 (solid square) and the high levels pH 8 (solid diamond), pH 9 (solid upright triangle) and pH 10 (solid hexagon) showed no binding.
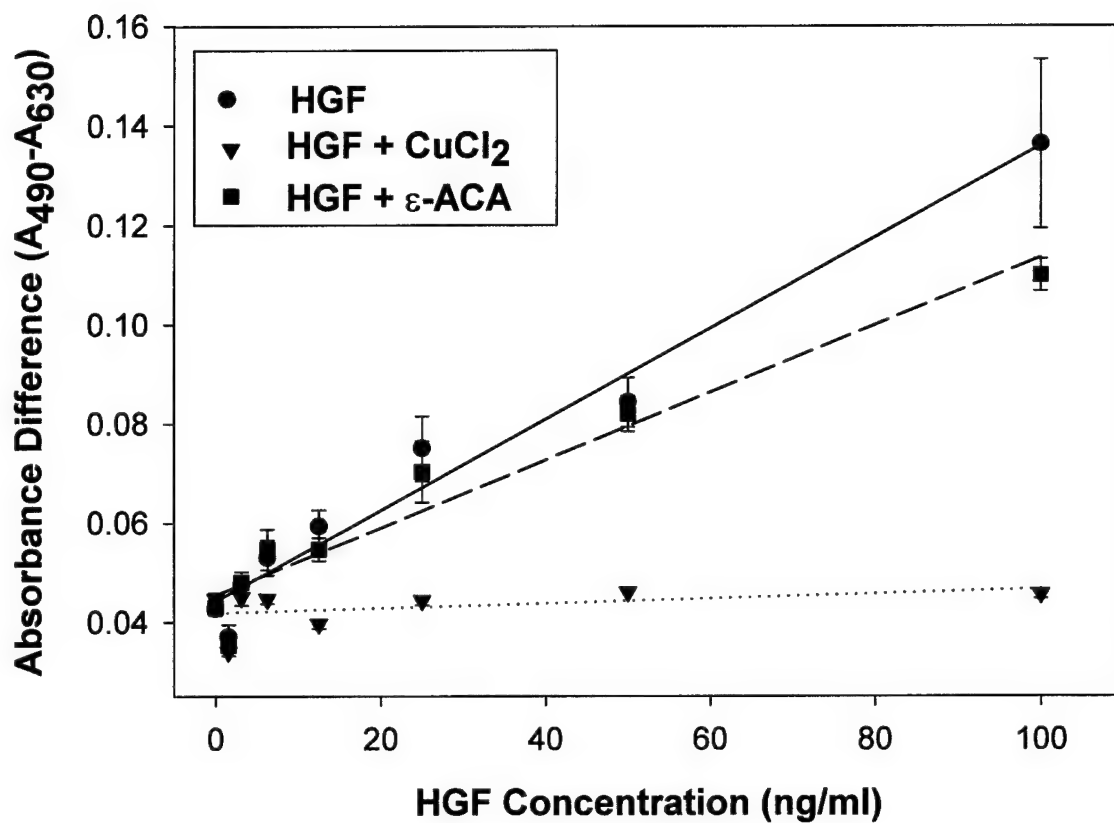
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Figure 6. Effect of 100 mM CuCl$_2$ and 100 mM ε-ACA on HGF binding to immobilized Met-IgG. Met-IgG was immobilized on an ELISA plate, followed by incubation with HGF alone (solid circles), in the presence of 100 mM CuCl$_2$ (solid upside-down triangles) or 100 mM ε-ACA (solid squares). HGF binding was measured by detecting HGF with a monoclonal anti-HGF IgG, followed by anti-murine HRP IgG and colour detection. CuCl$_2$ abolished binding, while ε-ACA had no effect.
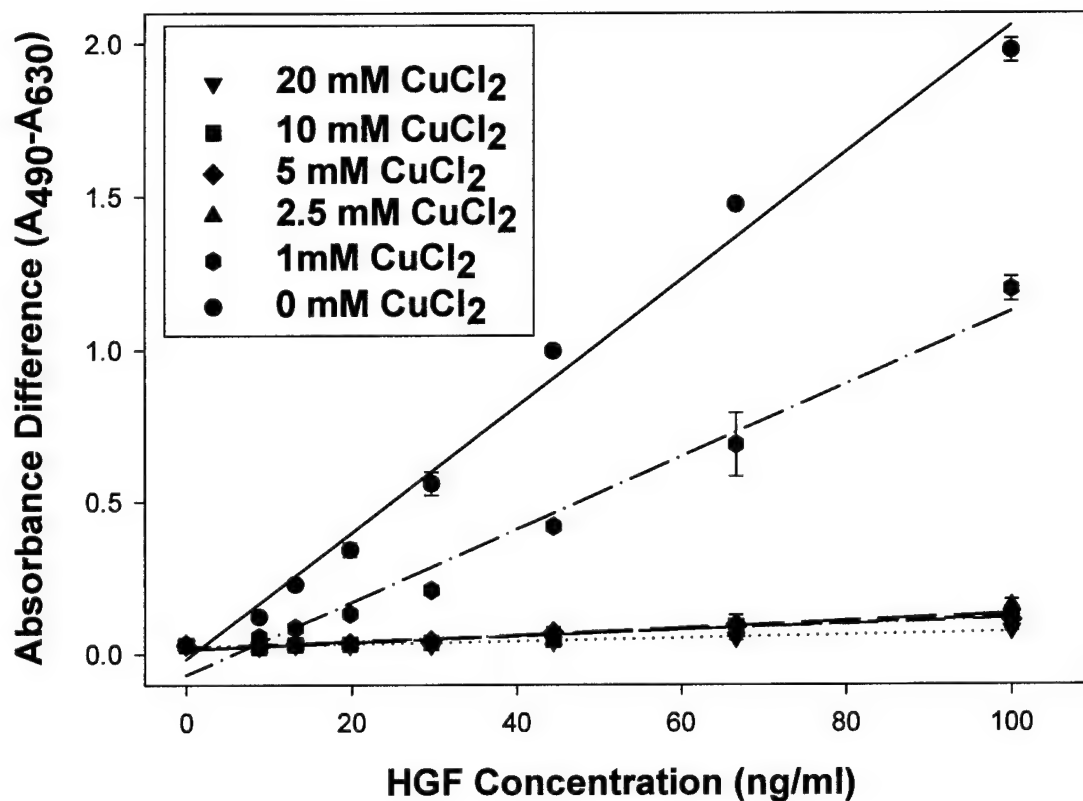
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Figure 7. Effect of various concentrations of $CuCl_2$ on HGF binding to immobilized Met-IgG. Met-IgG was immobilized on an ELISA plate, followed by incubation with HGF alone (solid circles), in the presence of 1 mM $CuCl_2$ (solid hexagons), 2.5 mM $CuCl_2$ (solid upright triangles), 5 mM $CuCl_2$ (solid diamonds), 10 mM $CuCl_2$ (solid squares) or 20 mM $CuCl_2$ (solid upside-down triangles). HGF binding was measured by detecting HGF with a monoclonal anti-HGF IgG, followed by anti-murine HRP IgG and colour detection. All $CuCl_2$ concentrations tested abolished binding, except 1 mM $CuCl_2$ which only partially inhibited binding.
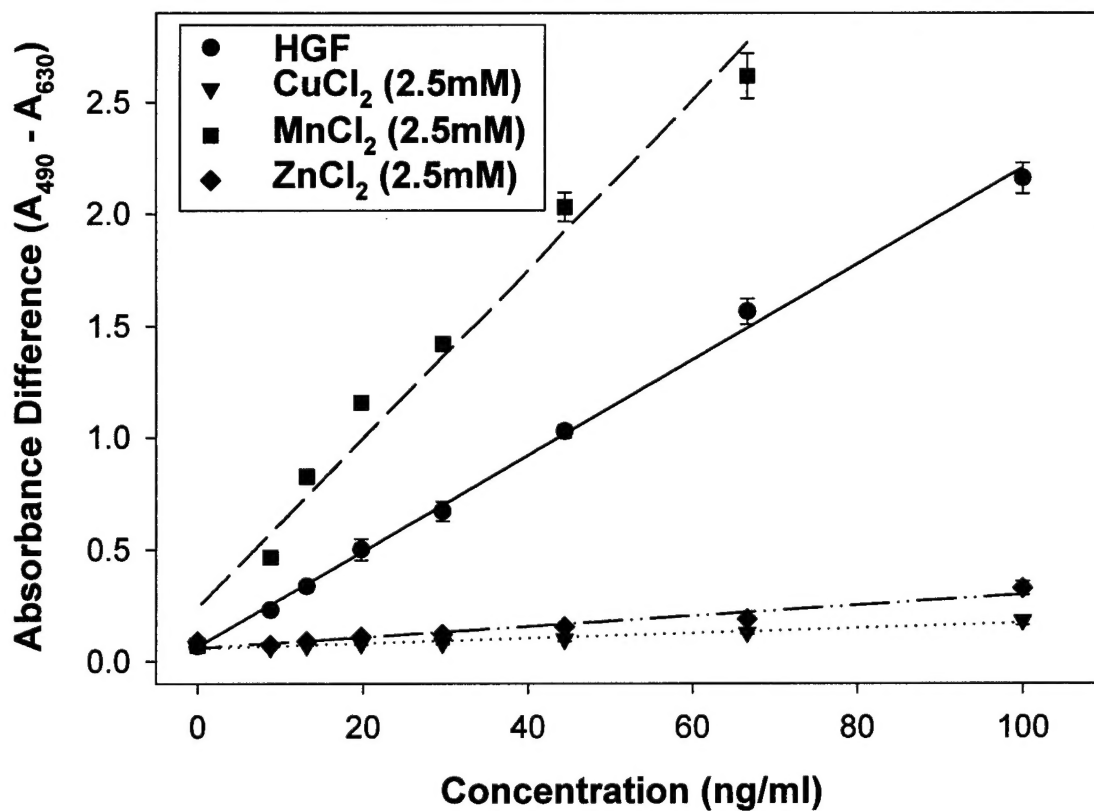
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Figure 8. Effect of 2.5 mM CuCl₂, 2.5 mM MnCl₂, and 2.5 mM ZnCl₂ on HGF binding to immobilized Met-IgG. Met-IgG was immobilized on an ELISA plate, followed by incubation with HGF alone (solid circles), in the presence of 2.5 mM CuCl₂ (solid upside-down triangles), 2.5 mM MnCl₂ (solid squares) or 2.5 mM ZnCl₂ (solid diamonds). HGF binding was measured by detecting HGF with a monoclonal anti-HGF IgG, followed by anti-murine HRP IgG and colour detection. CuCl₂ and ZnCl₂ abolished binding, while MnCl₂ enhanced binding.
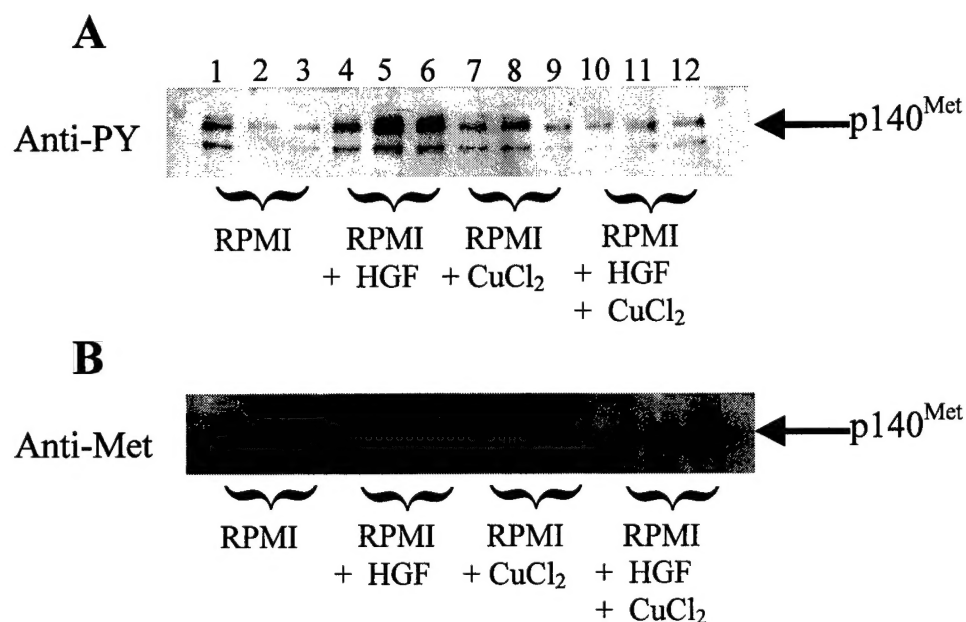
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Figure 9. Effect of 1 mM CuCl₂ on HGF-induced Met phosphorylation in SP1 cells.
SP1 cells were incubated in medium alone (lanes 1 to 3), in the presence of 20 ng/ml HGF (lanes 4 to 6), 1 mM CuCl₂ (lanes 7 to 9), or 20 ng/ml HGF and 1 mM CuCl₂ (lanes 10 to 12). Addition of HGF induced tyrosine phosphorylation of Met in SP1 cells, while CuCl₂ alone did not. In the presence of CuCl₂, HGF-induced tyrosine phosphorylation of Met was inhibited.
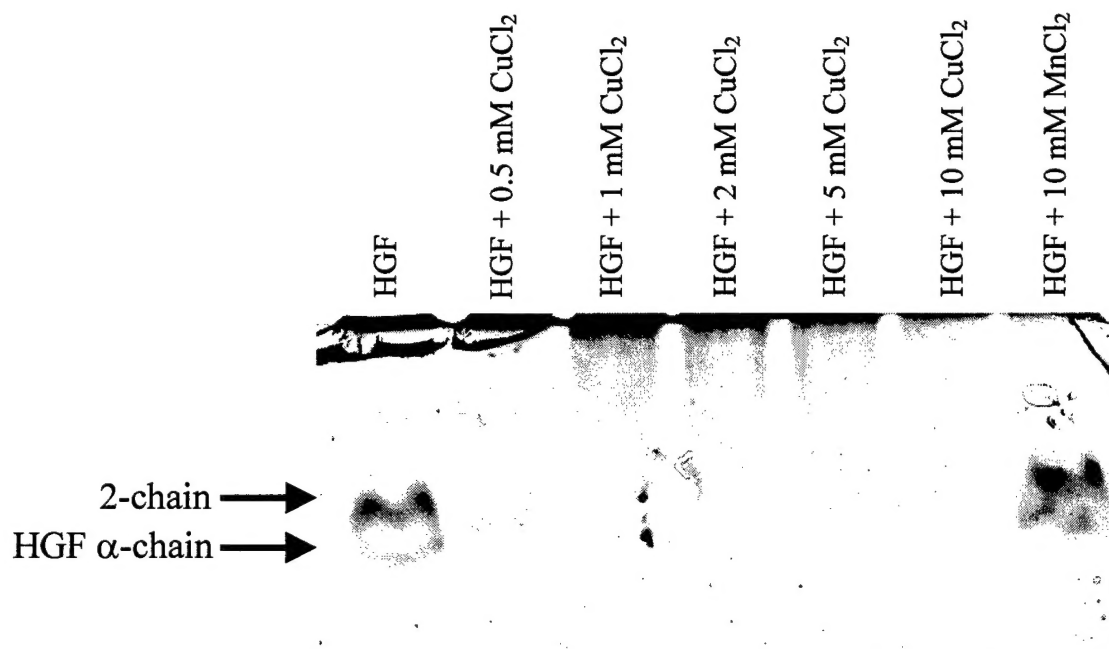
Figure 10. Mobility of rHGF-under non-denaturing conditions in the presence of CuCl$_2$. rHGF (5 μg) was incubated at 37°C for 2 hrs in Tris buffer alone (lane 1), or in Tris buffer containing 0.5 mM CuCl$_2$ (lane 2), 1 mM CuCl$_2$ (lane 3), 2 mM CuCl$_2$ (lane 4), 5 mM CuCl$_2$ (lane 5), 10 mM CuCl$_2$ (lane 6), or 10 mM MnCl$_2$ (lane 7). The mobility of rHGF alone or in the presence of MnCl$_2$ was similar, but rHGF mobility was modified by addition of as little as 0.05 mM CuCl$_2$. This indicates that Cu(II) causes a change in HGF mobility, whereas Mn(II) does not greatly affect HGF mobility.

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HEPATOCYTE GROWTH FACTOR INTERACTION WITH ITS RECEPTOR, MET

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Malignant breast carcinoma cells are highly motile and invasive and can lead to tumor spreading and metastasis. Hepatocyte growth factor (HGF) has been implicated in the increased invasiveness of breast cancer cells. Pro-HGF is secreted as a 110 kDa precursor chain, which is processed to an active heterodimer consisting of a 69 kDa α-chain and a 34 kDa β-chain. The α-chain of HGF contains a hairpin loop followed by four kringle domains. These kringles, of which the first two yield the greatest contribution, have been implicated in binding of HGF to its receptor, Met. Met is a heterodimer consisting of a 145 kDa transmembrane β-chain, containing the tyrosine kinase domain, and an extracellular 50 kDa α-chain. HGF normally interacts with Met on the surface of breast epithelial cells in a paracrine manner. In some mammary carcinoma cells an HGF/Met autocrine loop is established, leading to growth autonomy and increased invasiveness.

Our work has focused on the disruption of the interaction between HGF and Met, as a possible approach to therapeutic intervention in breast cancer. We have utilized an enzyme linked immunosorbant assay (ELISA) to observe HGF/Met binding, and potential disruption of this interaction. To study HGF/Met interactions in a cell-free system we have used a soluble chimeric form of Met, Met-IgG. Binding of HGF to Met-IgG was detected using a monoclonal anti-HGF antibody. Met-IgG or HGF was immobilized on an ELISA plate followed by incubation with the corresponding complementary protein. Potential inhibitors of HGF/Met-IgG binding were screened utilizing these assays.

Our results showed a linear HGF/Met-IgG binding relationship with increasing concentrations of HGF. Control human IgG showed very little non-specific binding. Addition of a polyclonal anti-Met antibody inhibited HGF/Met-IgG binding, in contrast to a polyclonal anti-HGF which did not inhibit HGF/Met-IgG binding. The anti-Met antibody may serve as potential inhibitor of HGF/Met binding, yielding information on important binding sites on Met. Co-incubation of HGF with a 0.1 M concentration of the lysine analogue ϵ-ACA did not affect binding to Met-IgG, indicating that, unlike other kringle proteins, binding of HGF to Met-IgG is not lysine-dependent.

Our laboratory has previously developed a method of HGF purification utilizing a Cu^{2+} chelating column. We therefore examined the effect of Cu^{2+} on HGF function. Co-incubation of 0.1 M Cu^{2+} with HGF caused complete abrogation of binding to Met-IgG. Prior incubation of HGF with Met-IgG, and subsequent addition of Cu^{2+}, did not disrupt binding. Taken together, these results indicate that Cu^{2+} binds to HGF and prevents its association with Met-IgG, but does not cause dissociation of HGF from the receptor. Also, addition of 500 μM Cu^{2+} inhibited HGF-induced scatter of MDCK cells, with no effect on cell viability. This non-toxic level of Cu^{2+} may reduce HGF dependent cell motility. Our results indicate that the HGF/Met interaction in breast carcinoma cells is a potential target site for inhibiting cell motility, invasiveness and metastasis.